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Cell Biology

Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: Evidence for more than one receptor class

(sequence analysis/baby hamster kidney cells/transfection/phosphorylation)

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ABSTRACT **The complete nucleotide sequence of a cDNA encoding the human platelet-derived growth factor (PDGF) receptor is presented. The cDNA contains an open reading frame that codes for a protein of 1106 amino acids. Comparison to the mouse PDGF receptor reveals an overall amino acid sequence identity of 86%. This sequence identity rises to 98% in the cytoplasmic split tyrosine kinase domain. RNA blot hybridization analysis of poly(A)⁺ RNA from human dermal fibroblasts detects a major (≈5.7 kb) and a minor (≈4.8 kb) transcript using the cDNA as a probe. Baby hamster kidney cells, transfected with an expression vector containing the receptor cDNA, express an ≈190-kDa cell surface protein that is recognized by an anti-human PDGF receptor antibody. The recombinant PDGF receptor is functional in the transfected baby hamster kidney cells as demonstrated by ligand-induced phosphorylation of the receptor. Binding properties of the recombinant PDGF receptor were also assessed with pure preparations of BB and AB isoforms of PDGF (i.e., PDGF dimers composed of two B chains or an A and a B chain). Unlike human dermal fibroblasts, which bind both isoforms with high affinity, the transfected baby hamster kidney cells bind only the BB isoform of PDGF with high affinity. This observation is consistent with the existence of more than one PDGF receptor class.**

Human platelet-derived growth factor (PDGF), a potent mitogen for mesenchymal cells in culture, was originally identified (1, 2) and purified from human platelets. Pure PDGF was described as a 27- to 31-kDa glycoprotein (3–6) thought to exist as a dimer of two distinct but highly similar polypeptides, termed A chain and B chain (7, 8). Although the exact composition of PDGF from human platelet sources with respect to its dimer composition is unclear, it is considered to be largely a heterodimer of A and B chains (PDGF-AB). More recently, homodimers of PDGF composed of two A chains (PDGF-AA) or two B chains (PDGF-BB) have been isolated from osteosarcoma cells and porcine platelets, respectively (9, 10).

The biological events triggered by PDGF (reviewed in ref. 11) are thought to be transmitted by means of a high-affinity cell surface receptor (12). This receptor has been described as a membrane glycoprotein of 160–185 kDa (13–15) that is tyrosine-phosphorylated in response to binding of PDGF (16–19) and possesses intrinsic tyrosine kinase activity (20). A cDNA encoding the mouse PDGF receptor has recently been cloned and sequenced, and its primary structure has been shown to be closely related to the v-*kit* oncogene and the receptor for macrophage colony-stimulating factor (21).

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We report here the nucleotide sequence and deduced amino acid sequence of a cDNA encoding the human PDGF receptor.§ We have expressed the cDNA coding for the human PDGF receptor in baby hamster kidney (BHK) cells and present evidence that these transfectants express a high-affinity receptor specific for the BB isoform of PDGF.

EXPERIMENTAL PROCEDURES

cDNA Library Screening. Two cDNA libraries, primed with random hexamers or oligo(dT), were prepared from poly(A)⁺ RNA from human dermal fibroblast cells (SK5) with *Eco*RI linkers and were cloned into phage λgt11, as described elsewhere (22, 23). Three oligonucleotide probes (see Fig. 1), 41, 45, and 38 nucleotides long, complementary to sequences of the mouse PDGF receptor [mouse nucleotides 310–350, 2353–2397, and 3391–3428, respectively (21)] were synthesized and used to screen the random-primed library. An *Sst* I/*Eco*RI fragment [630 base pairs (bp)] derived from the 3' end of clone RP41 (see Fig. 1) was used to screen the oligo(dT)-primed library. Both libraries were screened according to standard procedures (24).

DNA Sequencing. Restriction fragments of the cDNAs were subcloned into plasmid pUC13 for restriction mapping and into phage M13mp18 and M13mp19 for DNA sequence determination. Sequencing was done by the dideoxy chain termination method (25) from overlapping restriction fragments by using deoxyadenosine 5'-[α-^{35}S]thiotriphosphate from New England Nuclear (26). Sequencing reactions were catalyzed by the Klenow fragment of *Escherichia coli* DNA polymerase I (Pharmacia) or modified T7 DNA polymerase (United States Biochemical, Cleveland). A universal M13 primer or synthetic oligonucleotides complementary to the sequence were used as primers.

Cell Culture and Transfection. Diploid human dermal fibroblast cells (SK5) were cultured in Dulbecco–Vogt-modified Eagle's medium supplemented with 1% calf serum. BHK-570 cells descended from clone BHK 21-13 (27) and were cultured in Dulbecco–Vogt-modified Eagle's medium supplemented with 5% fetal bovine serum. Transfections of BHK-570 cells were performed using the calcium phosphate precipitation method (28). An expression vector (pDX) (29, 30) containing the human PDGF receptor cDNA (phPDGFR) (see Fig. 1) was cotransfected with a plasmid containing the methotrexate-resistant form of the dihydrofolate reductase gene into BHK-

Abbreviations: PDGF, platelet-derived growth factor; PDGF-AB, -AA, -BB, PDGF dimers composed of an A and a B chain, two A chains, or two B chains, respectively.

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Intelligenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03278).

570 cells (31). The transfected cells (BHK-c5, BHK-c6, and BHK-c8 cells) were placed under selection with 250 nM methotrexate. Surviving cell colonies were picked and assayed for the expression of the human PDGF receptor with a previously described anti-human PDGF receptor monoclonal antibody (PR7212) (32) that does not recognize rodent PDGF receptors.

Saturation Binding Assay. Test cells were plated in 24-well trays at 3×10^4 cells per well in the appropriate culture medium. Three days after plating, the medium was removed, the cells were washed once with 1 ml per well of phosphate-buffered saline at 4°C, and a saturation binding assay was performed by using increasing concentrations of ^{125}I-labeled antibody PR7212, PDGF-BB, or PDGF-AB, essentially as described (33). PDGF-BB was labeled using the Bolton and Hunter reagent (ICN). Calculations of the apparent K_d were performed as described (33). For these studies we have used purified recombinant PDGF-BB homodimers synthesized by a *Saccharomyces cerevisiae* expression system (34). PDGF-AB heterodimers were purified from human platelet-rich plasma by immunoaffinity chromatography using specific monoclonal antibodies against PDGF-AB and PDGF-BB (C.E.H., unpublished results).

Phosphorylation and Radioimmunoprecipitation. Membrane extracts of SK5 cells, BHK-570 cells, and BHK-c6 cells were prepared as previously described (32). Membrane extracts from 5×10^6 cells were incubated for 30 min on ice with 200 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; Du Pont–New England Nuclear) in the presence or absence of 10 nM PDGF-BB. The phosphorylated membrane extracts were then immunoprecipitated with the antibody PR7212 or a negative control antibody (32). The precipitated samples were separated by NaDodSO$_4$/PAGE under reducing conditions; the gel was dried and autoradiographed at −70°C.

RNA Blotting and Hybridization. Total cellular RNA was extracted using a modification of the guanidinium method (35). In some cases this was followed by oligo(dT)-cellulose chromatography (36) to obtain poly(A)$^+$ RNA. After electrophoresis in 1% agarose gels containing formaldehyde, the RNA was transferred to nylon filters, prehybridized for 4 hr, and hybridized overnight at 47°C under standard conditions (37) with a 4.7-kilobase (kb) cDNA *Eco*RI/*Xba* I fragment of the phPDGFR cDNA insert (Fig. 1) as a probe. The probe was labeled with [α-^{32}P]dCTP by using a random oligonucleotide labeling kit (Pharmacia). Filters were subsequently washed in 0.05 M sodium phosphate/1 mM EDTA/1% NaDodSO$_4$ at 65°C.

RESULTS

Isolation of cDNA Clones Coding for the Human PDGF Receptor. Approximately 1×10^6 phage from the random-primed human dermal fibroblast (SK5) cDNA library were screened with a mixture of three unique oligonucleotide probes. Eight positive clones were identified and plaque purified. Two clones, designated RP41 and RP51 (Fig. 1), were selected for further analysis by restriction enzyme mapping and DNA sequencing. Clone RP51 contains 1097 nucleotides, which includes 356 nucleotides of 5'-noncoding sequence, the ATG translation initiation codon, and 738 nucleotides of the amino-terminal coding sequence. Clone RP41 overlaps clone RP51 and consists of 2649 nucleotides coding for the central portion of the protein (amino acids 43–925, Fig. 2). Since the eight clones isolated from the random-primed library did not extend to the 3' end of the full-length cDNA, approximately 0.6×10^6 phage of the oligo(dT)-primed SK5 cDNA library were screened to obtain clones coding for this region. Clone OT91, one of 10 positive clones, contains 2856 nucleotides including the 3' end of the coding region and 1896 nucleotides of 3'-untranslated sequence, but no poly(A)$^+$ tail or polyadenylylation signal were found (Fig. 1). To reconstruct a cDNA coding for the entire PDGF receptor, clones RP51, RP41, and OT91 were ligated together at common *Acc* I and *Bam*HI sites. The resulting cDNA, designated phPDGFR (Fig. 1), was subcloned into a eukaryotic expression vector (pDX) (29) for subsequent transfection.

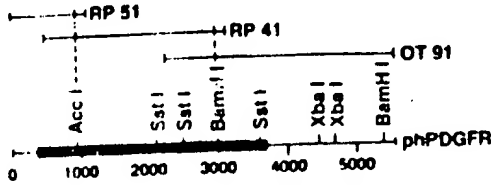

FIG. 1. Partial restriction map and cloning strategy for the human PDGF receptor cDNA. Three clones, RP41, RP51, and OT91, were used to reconstruct a cDNA, designated phPDGFR, encoding the human PDGF receptor. The clones and the derived cDNA are depicted without *Eco*RI linkers on both ends. The coding region is shown as a solid black bar. Dashed lines indicate the restriction sites used to ligate the partial clones together. A scale (in nucleotides) is shown below phPDGFR.

Nucleotide Sequence and Deduced Amino Acid Sequence of the Human PDGF Receptor. The nucleotide sequence of the cDNA encoding the human PDGF receptor (phPDGFR) consists of 5570 nucleotides and predicts a protein of 1106 amino acids (Fig. 2). The amino acid sequence of this protein is very similar to the mouse PDGF receptor as demonstrated by an overall sequence identity of 86%. By comparison with the domain structure that has been described for the mouse PDGF receptor (21), the sequence identities to the human receptor are as follows: signal peptide, 63%; extracellular domain, 79%; transmembrane domain, 96%; split tyrosine kinase domain, 98%; and the remaining intracellular domain, 85%. Additional similarities between the two sequences include conservation of (*i*) the number and position of all cysteine residues (with the exception of Cys-787) and (*ii*) 10 of 11 potential asparagine-linked glycosylation sites in the extracellular portion of the sequence.

On the nucleotide level, comparison of the mouse and human cDNAs shows overall identities of 72% in the coding region and 65% in the 5'- and 3'-untranslated regions. Several more highly conserved regions are also found at both the 5' and 3' ends of the cDNA. For example, the sequence ATTTA (Fig. 2), associated with selective mRNA degradation (38), is found at the 3' end of the mouse and human cDNAs in an A+T-rich region that is 85% identical over a stretch of 150 bases.

Expression of the PDGF Receptor in BHK Cells. To confirm that the phPDGFR sequence (Fig. 1) coded for a functional receptor, the cDNA was transfected into BHK-570 cells, which do not express a detectable amount of endogenous PDGF receptor (see Figs. 3 and 5). Expression of the recombinant PDGF receptor in these transfectants was evaluated by binding of a monoclonal anti-receptor antibody, PR7212, and pure preparations of the PDGF-BB and PDGF-AB ligands. In addition, we examined the phosphorylation of the expressed receptor in response to ligand binding.

Binding studies were performed with three transfectants, BHK-c5, BHK-c6, and BHK-c8. Parental BHK-570 cells and human dermal fibroblasts (SK5) were used as negative and positive controls, respectively. At saturating concentrations, the binding of antibody PR7212 to the three transfectants was 1.5-, 2.6-, and 5-fold greater than to SK5 cells (Fig. 3*A*). Binding of ^{125}I-labeled PDGF-BB (Fig. 3*B*) revealed high-affinity binding sites on both the transfectants and the SK5 cells (apparent $K_d = 4–7 \times 10^{-11}$ M). Binding of PDGF-BB to BHK-c6 cells was not saturable in this experiment due to depletion of the ligand by the very high number of receptors

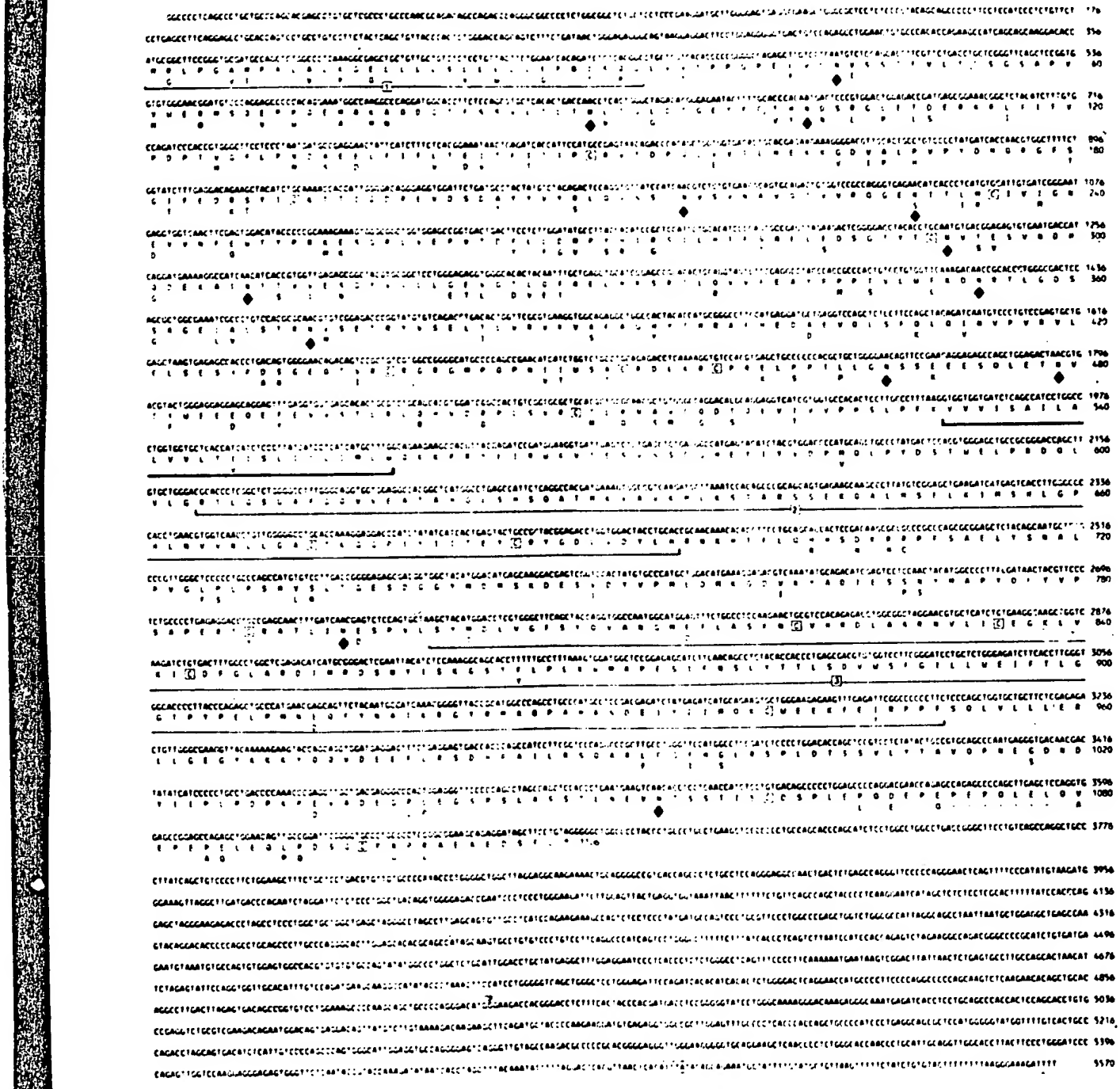

FIG. 2. Nucleotide sequence of the human PDGF receptor and comparison of the deduced amino acid sequence with the mouse PDGF receptor sequence (21). For maximal alignment of the mouse and human amino acid sequences, two gaps were inserted into the mouse sequence (positions 18 and 1072–1078). Nucleotides and amino acids of the human PDGF receptor are numbered at the right. Amino acid numbering starts at the N-terminal methionine. Residues of the mouse PDGF receptor that differ from the human sequence are depicted below the human amino acid sequence. Potential sites of asparagine-linked glycosylation are marked by solid diamonds below the asparagine residue. Cysteine residues are boxed. The putative transmembrane domain is underlined with a heavy line. Other domains are underlined and labeled with boxed numbers: signal peptide domain 1 and the split tyrosine kinase domain 2 and 3. An AUUUA sequence motif is demarcated at the 3′ end of the cDNA. The stop codon is indicated by an asterisk.

per culture. In other experiments, with lower cell densities, binding to BHK-c6 was saturable and showed an affinity comparable to the one obtained with SK5 cells. In contrast to the PDGF-BB binding, ^{125}I-labeled PDGF-AB (Fig. 3C) bound with high affinity only to SK5 cells (apparent K_d = 2 $\times$ 10^{-11} M) and with a much lower affinity to the transfectants. Among the transfectants, the amount of low-affinity binding of ^{125}I-labeled PDGF-AB (Fig. 3C) correlated with the amount of high-affinity binding of ^{125}I-labeled PDGF-BB and antibody PR7212 (Fig. 3 *A* and *B*).

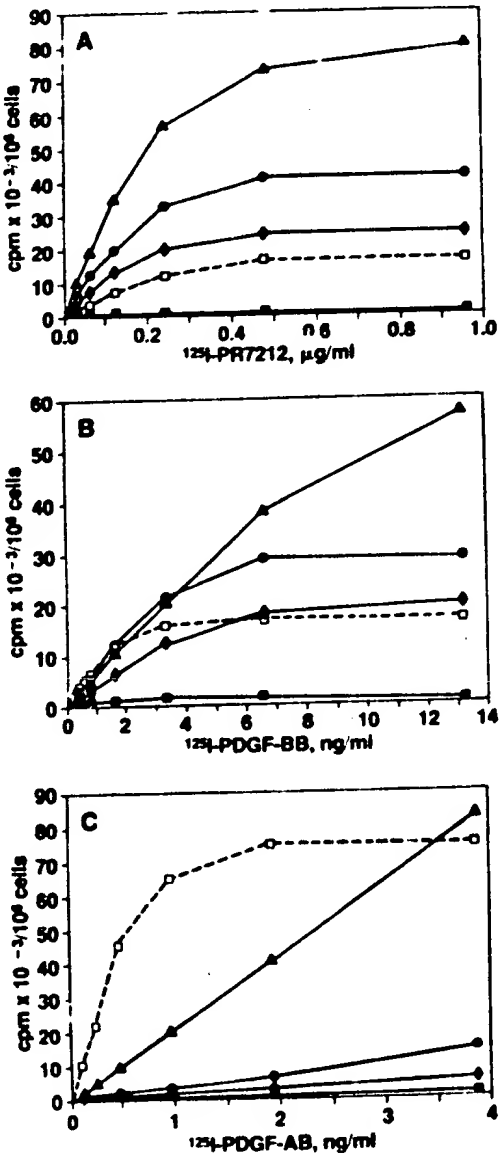

FIG. 3. Binding of antibody PR7212, PDGF-BB, and PDGF-AB to SK5 cells, BHK-570 cells, and to the transfectants BHK-c5, BHK-6, and BHK-8. Confluent cultures of cells were incubated in parallel with increasing concentrations of ^{125}I-labeled PR7212 (*A*), ^{125}I-labeled PDGF-BB (*B*), or ^{125}I-labeled PDGF-AB (*C*). The symbols represent triplicate measurements of the following cell types: ■, BHK-570; ◆, BHK-c5; ▲, BHK-c6; ●, BHK-c8; □, SK5. Nonspecific binding was determined as described (33) and was subtracted from the individual data points. Receptor numbers were not calculated because the true concentration and specific activity of the ligand were not precisely known.

Radioimmunoprecipitation of Phosphorylated PDGF Receptors. It has previously been shown that the PDGF receptor is phosphorylated in response to binding of PDGF (16–19). To investigate whether the recombinant PDGF receptor in baby hamster kidney cells also shows stimulation of phosphorylation in response to ligand binding, we prepared membrane extracts and examined the level of phosphorylation of the recombinant PDGF receptor in the presence and absence of PDGF-BB. PDGF-BB stimulated easily detectable phosphorylation of ≈160- and ≈190-kDa proteins in crude membrane

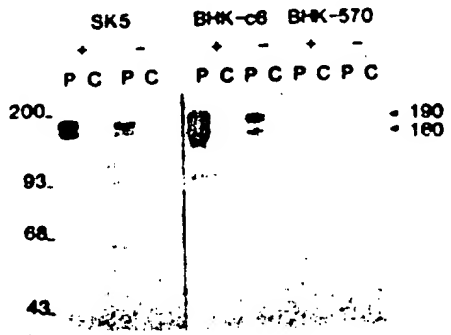

FIG. 4. Immunoprecipitation of [γ-^{32}P]ATP-labeled membrane extracts using antibody PR7212. Membrane extracts of the indicated cells were prepared, and aliquots equivalent to 5×10^6 cells were then incubated for 30 min in the presence (+) or absence (−) of 10 nM PDGF-BB. Subsequent to gel filtration, the extracts were immunoprecipitated using antibody PR7212 (lanes P) or a control monoclonal antibody (lanes C). Positions of the molecular size markers (in kDa) are indicated at the left. The arrowheads at the right indicate the position of the ≈160- and ≈190-kDa proteins.

preparations (data not shown). Fig. 4 shows the basal and stimulated levels of phosphorylation of the PDGF receptor in immunoprecipitates of *in vitro* phosphorylated membranes from SK5, BHK-c6, and parental BHK-570 cells. In BHK-c6 cells the addition of PDGF-BB induced phosphorylation of proteins of ≈160 kDa and ≈190 kDa. In the control SK5 cells a similar induction of ≈164-kDa and ≈180-kDa bands could be detected. The size of these proteins agrees well with the previously reported sizes of ≈164 kDa for the precursor and ≈180 kDa for the mature PDGF receptor in human fibroblast cells (32). As expected, no phosphorylation of PDGF receptors was detected in BHK-570 cells.

RNA Blot Hybridization Analysis. To estimate the size and relative abundance of the PDGF receptor transcript in SK5 human fibroblasts and in the transfected BHK cells, RNA blot hybridizations were performed using a 4.7-kb *Eco*RI/*Xba* I fragment of phPDGFR as a probe (Fig. 1). Two transcript sizes (≈5.7 kb and ≈4.8 kb) could be detected in oligo(dT)-selected RNA (Fig. 5, lane d) from SK5 cells. When total RNA was used, the migration of the ≈4.8-kb band was apparently accelerated by large amounts of 28S RNA (Fig. 5, lane c). The ≈5.7-kb band was estimated to be 10-fold more intense than the ≈4.8-kb band. Total cellular RNA prepared from both A431 human epidermoid carcinoma cells and BHK-570 cells showed no hybridization signal (Fig. 5, lanes a and b). When exposed for the same length of time as lanes a–d in Fig. 5 (24 hr at −70°C), the signal from the transfectant BHK-c6 cells (Fig. 5, lane e) was greatly overexposed, indicating much greater mRNA abundance in the BHK-c6 transfectants compared to SK5 cells. This is consistent with the greater level of receptor expression in these transfectants.

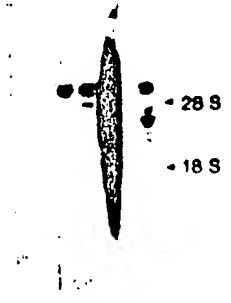

FIG. 5. RNA blot hybridization analysis. Total and oligo(dT)-selected mRNA were isolated. Lanes: a, 20 μg of total A431 RNA; b, 20 μg of total BHK-570 RNA; c, 10 μg of total SK5 RNA; d, 2 μg of poly(A) SK5 RNA; e, 20 μg of total BHK-c6 RNA. Lane f represents a 1-hr exposure of lane e. Exposure time for lanes a–e was 24 hr at −70°C with an intensifying screen. rRNA bands were used as size references [4760 bp (28S) and 1780 bp (18S)].

A shorter exposure (1 hr at −70°C) yielded a major band of ≈5.7 kb (Fig. 5, lane f). This size estimate is close to the 5.9-kb size expected for the PDGF receptor transcript generated by the expression vector pDX. The nature of the lower molecular mass band detected in Fig. 5, lane f is unknown.

DISCUSSION

We report here the cloning, sequence, and expression of a cDNA encoding the human PDGF receptor. We have compared this human cDNA sequence and its deduced amino acid sequence to that of the mouse PDGF receptor published by Yarden *et al.* (21). The nucleotide sequences are similar throughout their length, including segments of high sequence similarity in the 5'- and 3'-untranslated regions. The deduced amino acid sequence of the human PDGF receptor is also very similar to the mouse sequence and, hence, exhibits the same functional domains as those described for the mouse PDGF receptor (21). These putative domains include a signal peptide, an extracellular domain, a transmembrane domain, and a cytoplasmic domain containing a split tyrosine kinase domain. From the extensive degree of sequence similarity to the mouse receptor, we conclude that, most likely, we have cloned the human version of the PDGF receptor.

The human cDNA has been used to probe human RNA in order to detect the pattern of PDGF receptor transcripts. In human dermal fibroblasts, we detect transcripts of ≈5.7 and ≈4.8 kb. This is in contrast to a single ≈5.3 kb transcript reported by Yarden *et al.* (21). The two transcripts reported here are detected under stringent hybridization conditions in which there is no detectable hybridization to RNA isolated from either human A431 cells or BHK-570 cells. The larger and more intense band (≈5.7 kb) is similar in size to the 5570 nucleotide human cDNA reported here, indicating that we have essentially cloned the entire cDNA. The origin of the smaller less intense ≈4.8 kb band is unclear, but it could arise as a result of alternative splicing or the use of an atypical alternative polyadenylylation signal. Finally, the ≈4.8-kb band could be derived from a second distinct locus.

When the human PDGF receptor cDNA is expressed in BHK-570 cells, we find that the transfected cells are now able to bind the anti-PDGF receptor antibody PR7212 as well as the PDGF-BB ligand. Furthermore, ligand-induced phosphorylation of two membrane proteins that are immunoprecipitable by antibody PR7212 could be demonstrated. The sizes of the two receptor proteins found in the transfectants correspond well to those seen in the human dermal fibroblasts. These results indicate that the transfectants express at least a partially functional PDGF receptor. Experiments determining whether the transfected BHK cells respond mitogenically to PDGF are needed.

Recent evidence (39) has suggested the existence of at least two PDGF receptor classes. One receptor class (termed the "B receptor") binds only PDGF-BB, and the other (termed the "A receptor") binds all three isoforms of PDGF (AA, AB, and BB). The observation presented in this manuscript that the transfectants bind only PDGF-BB with high-affinity is consistent with the characteristics of the predicted B receptor class. Several explanations for this result are possible. The most direct is that we have cloned and expressed a cDNA coding for one of the proposed PDGF receptor classes (the B receptor) and that a second distinct gene product for the other PDGF receptor class exists. Alternatively, posttranslational modifications could be responsible for generating two classes of PDGF receptors, and BHK-570 cells might lack this ability. Finally, an accessory protein may exist in human dermal fibroblasts but not in BHK-570 cells, which is required for high-affinity binding of PDGF-AB to the PDGF receptor. A situation similar to this possibility has been shown in the interleukin 2 receptor system (40).

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Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types

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The binding of the three dimeric forms of platelet-derived growth factor (PDGF), PDGF-AA, PDGF-AB and PDGF-BB, to human fibroblasts was studied. Cross-competition experiments revealed the existence of two different PDGF receptor classes: the type A PDGF receptor bound all three dimeric forms of PDGF, whereas the type B PDGF receptor bound PDGF-BB with high affinity and PDGF-AB with lower affinity, but not PDGF-AA. The sizes of the two receptors were estimated with affinity labeling techniques; the A type receptor appeared as a major component of 125 kd and a minor of 160 kd, and the B type receptor as two components of 160 and 175 kd. A previously established PDGF receptor monoclonal antibody, PDGFR-B2, was shown to react with the B type receptor only. The different abilities of the three dimeric forms of PDGF to stimulate incorporation of [^{3}H]TdR into human fibroblasts indicated that the major mitogenic effect of PDGF is mediated via the B type receptor.

Key words: platelet-derived growth factor/receptor/human fibroblasts

Introduction

Platelet-derived growth factor (PDGF) is a major mitogen for connective tissue cells *in vitro* (reviewed in Heldin *et al.*, 1985; Ross *et al.*, 1986). The functionally active molecule is made up as a dimer of two polypeptide chains that are linked by disulphide bonds. The subunit chains occur in two different forms, denoted A and B, and their mature parts show 60% amino acid sequence similarity with perfect conservation of all eight cysteine residues (Johnsson *et al.*, 1984; Josephs *et al.*, 1984; Betsholtz *et al.*, 1986).

All three possible dimeric forms of PDGF have been identified: PDGF purified from human platelets is a heterodimer, PDGF-AB (Hammacher *et al.*, 1988a); PDGF-AA has been found in media conditioned by human osteosarcoma (Heldin *et al.*, 1986), melanoma (Westermark *et al.*, 1986) and glioma (Hammacher *et al.*, 1988b) cell lines, and is probably produced by rat smooth muscle cells (Sejersen *et al.*, 1986) and mitogen-stimulated human fibroblasts (Paulsson *et al.*, 1987); a factor similar to PDGF-BB exerts the transforming activity of simian sarcoma virus (Waterfield *et al.*, 198. Doolittle *et al.*, 1983; Robbins *et al.*, 1983), and in addition, PDGF purified from porcine platelets has the structure of a B chain homodimer (Stroobant and Waterfield, 1984).

It was recently found that the different dimeric forms of PDGF have different functional activities. Thus, PDGF-AA has a considerably lower mitogenic activity, chemotactic activity and ability to cause actin rearrangement, than has PDGF-AB (Nistér *et al.*, 1988); it was suggested that this is due to different reactivities with two distinct receptor classes. Indications of the existence of more than one PDGF receptor type also came from experiments demonstrating that the different dimers have different ability to down-regulate PDGF receptors (Hart *et al.*, 1988; Nistér *et al.*, 1988).

In this communication, we provide direct evidence for the presence of two distinct PDGF receptor types on human fibroblasts. The receptors are distinguished by their different ligand binding specificities, their sizes as estimated by affinity labeling, and by their different reactivities with a recently established monoclonal antibody against a PDGF receptor purified from porcine uterus (Rönnstrand *et al.*, 1988).

Results

Cross-competition for binding to human foreskin fibroblasts of different dimeric forms of PDGF

PDGF-AB purified from human platelets and recombinant PDGF A and B chain homodimers were ^{125}I-labeled and analysed with regard to their binding to human foreskin fibroblasts at 0°C. All three dimeric forms of PDGF showed a specific binding to the cells. As shown in Figure 1, [^{125}I]PDGF-AB was most efficiently competed for by PDGF-AB, with a 50% reduction in binding at 5 ng/ml, but PDGF-AA and PDGF-BB also competed with half-maximal effects at about 30 and 60 ng/ml respectively. Also, [^{125}I]-PDGF-AA was most efficiently competed for by PDGF-AB, with half-maximal effect at 5 ng/ml. In addition, PDGF-AA and PDGF-BB competed; half-maximal effects were achieved at 10 and 30 ng/ml respectively. In contrast, [^{125}I]PDGF-BB was only marginally competed for by PDGF-AA, whereas PDGF-AB and PDGF-BB competed with half-maximal effects at 40 and 60 ng/ml respectively.

These results indicate that at least two PDGF receptor classes exist on human fibroblasts. One receptor showed highest affinity for PDGF-AB, but bound also PDGF-AA and PDGF-BB. This receptor is tentatively called PDGF receptor type A. The other receptor bound PDGF-BB and PDGF-AB, but not PDGF-AA. This receptor is tentatively called PDGF receptor type B.

Binding of PDGF-AB and PDGF-BB to human fibroblasts after down-regulation with PDGF-AA

The result of the experiment described in Figure 1 indicates that PDGF-AB and PDGF-BB bind both to type A and type B receptors on human fibroblasts. In order to characterize

the binding of these ligands to the B type receptor only, the A type receptor was down-regulated by incubation of the fibroblasts with PDGF-AA for 1 h at 37°C. A wash at pH 3.75, which would dissociate bound PDGF-AA from the receptor, did not expose any A type receptor on such cells, indicating that the receptor was down-regulated rather than remaining at the cell surface but blocked by an excess of ligand (not shown). Dose–response analysis revealed that 50 ng/ml of PDGF-AA caused maximal down-regulation (not shown); this concentration was therefore used. After preincubation, the cells were cooled to 0°C and the binding of [^{125}I]PDGF-AB and [^{125}I]PDGF-BB determined. As shown in Figure 2A, preincubation with PDGF-AA resulted in a reduction of [^{125}I]PDGF-AB binding to about 25% of the control value. This effect was interpreted as resulting from a down-regulation of type A receptors. The binding of [^{125}I]PDGF-AB to the remaining B type receptor was of low affinity; 100 ng/ml of PDGF-AB reduced the binding of [^{125}I]PDGF-AB from 25 to 17%, i.e. by only 30% (Figure 2A). In contrast, as was expected from the experiment described in Figure 1, preincubation with PDGF-AA had very little effect on the binding of [^{125}I]PDGF-BB (Figure 2B). Thus, the B type receptor binds primarily PDGF-BB, and PDGF-AB with lower affinity.

Dissociation of [^{125}I]PDGF-AB and [^{125}I]PDGF-BB from human fibroblasts

Prebound [^{125}I]PDGF-AB has been found to dissociate very slowly from the surface of human fibroblasts upon incubation at 0°C (Heldin *et al.*, 1982). To investigate which one of the two PDGF receptor types is involved in the stabilization of binding, the dissociation of [^{125}I]PDGF-AB from human fibroblasts was compared with that from fibroblasts preincubated with PDGF-AA at 37°C to down-regulate the A type receptor. PDGF-AA pretreated cells and control cells were incubated with [^{125}I]PDGF-AB at 0°C, washed and further incubated at 0°C with increasing concentrations of unlabeled PDGF-AB; after 60 min of incubation the amount of [^{125}I]PDGF-AB released into the medium was determined. Only a small fraction (~20%) of bound [^{125}I]-PDGF-AB was displaced and there was essentially no effect of addition of unlabeled PDGF-AB to the medium, or by preincubation with PDGF-AA (Figure 3A). A similar experiment with [^{125}I]PDGF-BB gave essentially the same result; the displacement of [^{125}I]PDGF-BB was not affected by the addition of unlabeled PDGF-BB, nor by preincubation with PDGF-AA (Figure 3B). This indicates that the binding of

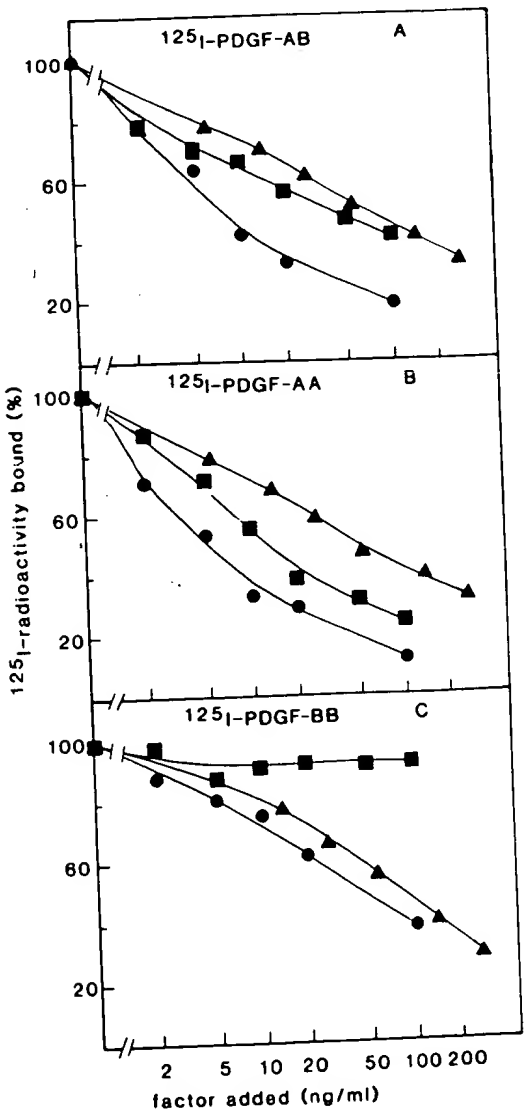

Fig. 1. Cross-competition for binding to human fibroblasts of different dimeric forms of PDGF. The binding of ^{125}I-labeled PDGF-AB (A), PDGF-AA (B) and PDGF-BB (C) to human fibroblasts at 0°C was determined in the presence of increasing concentrations of PDGF-AB (●—●), PDGF-AA (■—■) and PDGF-BB (▲—▲). The binding in the absence of unlabeled ligands (2400 c.p.m. [^{125}I]PDGF-AB; 2900 c.p.m. [^{125}I]PDGF-AA; 3200 c.p.m. [^{125}I]PDGF-BB) were set at 100%.

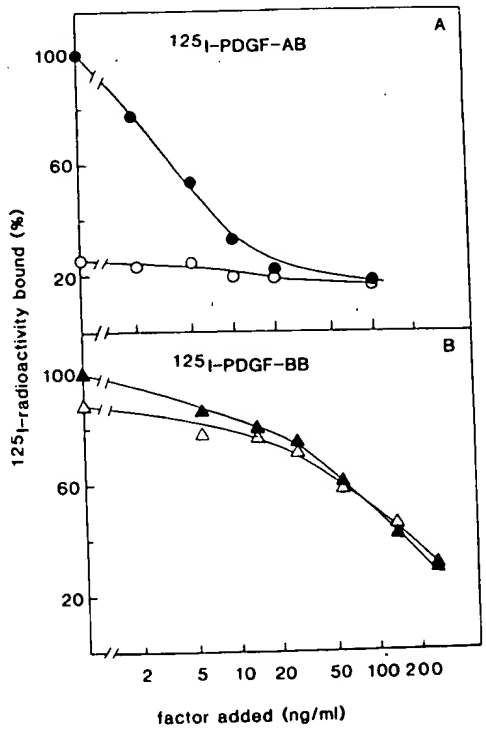

Fig. 2. Binding of PDGF-AB and PDGF-BB to human fibroblasts after down-regulation with PDGF-AA. The binding of [^{125}I]PDGF-AB (A) and [^{125}I]PDGF-BB (B) to human fibroblasts at 0°C was determined in the presence of increasing concentrations of the corresponding unlabeled ligands on cells incubated in the absence (closed symbols) or presence (open symbols) of 50 ng/ml PDGF-AA for 1 h at 37°C. 100% binding refers to 3000 c.p.m. [^{125}I]PDGF-AB and 4000 c.p.m. [^{125}I]PDGF-BB bound.

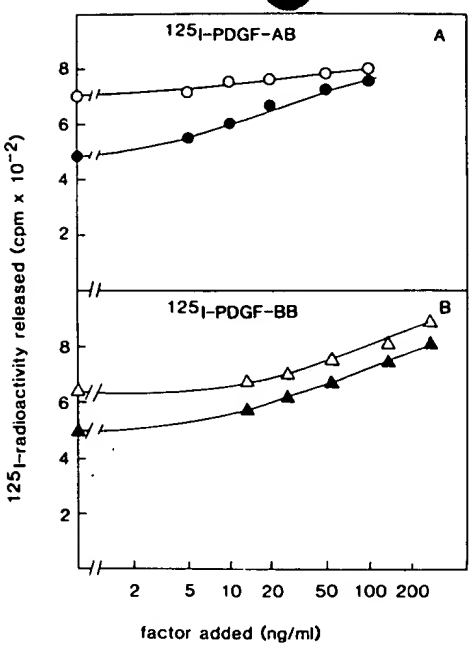

Fig. 3. Dissociation of [^{125}I]PDGF-AB and [^{125}I]PDGF-BB from human fibroblasts. [^{125}I]PDGF-AB (**A**) and [^{125}I]PDGF-BB (**B**) were bound for 2 h at 0°C to human fibroblasts that had been preincubated in the absence (closed symbols) or presence (open symbols) of 50 ng/ml PDGF-AA for 1 h at 37°C. After washing, incubation was continued at 0°C in the presence of increasing concentrations of the corresponding ligands; after 1 h the radioactivity released into the medium was determined. Total binding of [^{125}I]PDGF-AB was 2000 and 3500 c.p.m. on cells that were not preincubated with PDGF-AA respectively; the corresponding binding of [^{125}I]PDGF-BB was 1800 and 3000 c.p.m. respectively.

[^{125}I]PDGF-AB and [^{125}I]PDGF-BB to the B type receptor undergoes stabilization after binding, which then prevents their dissociation from the receptor.

Affinity cross-linking of different ^{125}I-labeled dimeric forms of PDGF

Affinity cross-linking using the homobifunctional cross-linker disuccinimidyl suberate (DSS) was used to characterize the sizes of the A and B types of PDGF receptors. The different ^{125}I-labeled dimeric forms of PDGF were bound to fibroblasts at 0°C; after washing, DSS was added to the cell cultures in order to cause covalent binding of the ligands to their receptors. Analysis by SDS–gel electrophoresis and autoradiography revealed that [^{125}I]PDGF-AA was cross-linked in a major complex of 140 kd and a minor one of 175 kd, [^{125}I]PDGF-BB was cross-linked to form complexes of 175–190 kd, whereas [^{125}I]PDGF-AB formed complexes of both 175–190 kd and 140 kd (Figure 4). In all cases, addition of an excess of the corresponding unlabeled ligand during incubation caused a decrease in the amount of radioactivity associated with high mol. wt components, indicating specific interactions between ligands and receptors.

The three ligands were also bound and cross-linked to cells that had been preincubated at 37°C with PDGF-AA, to remove the A type receptor. On these cells, [^{125}I]PDGF-AA was not cross-linked to any high mol. wt complex, whereas the cross-linking of [^{125}I]PDGF-BB to form the 175–190 kd complex remained (Figure 4). This is consistent with the conclusion that the 140 kd component contains the A type receptor and the 175–190 kd component(s) the B type receptor.

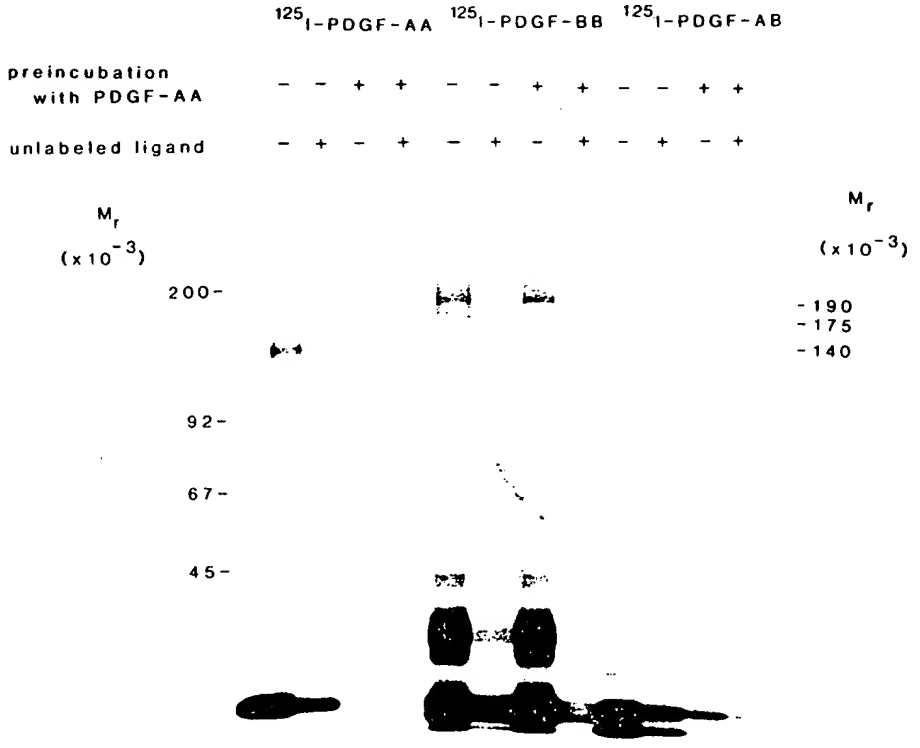

Fig. 4. Affinity cross-linking of different ^{125}I-labeled dimeric forms of PDGF to human fibroblasts. [^{125}I]PDGF-AB, [^{125}I]PDGF-AA and [^{125}I]PDGF-BB were incubated for 2 h at 0°C with cells that had been preincubated for 1 h at 37°C with or without 50 ng/ml PDGF-AA. After washing, the ^{125}I-labeled ligands were cross-linked with 0.05 mM DSS for 30 min at 0°C; samples were analysed by SDS–gel electrophoresis and autoradiography. As a control of specificity the bindings were performed in the absence or presence of 50 ng/ml of the corresponding unlabeled ligands.

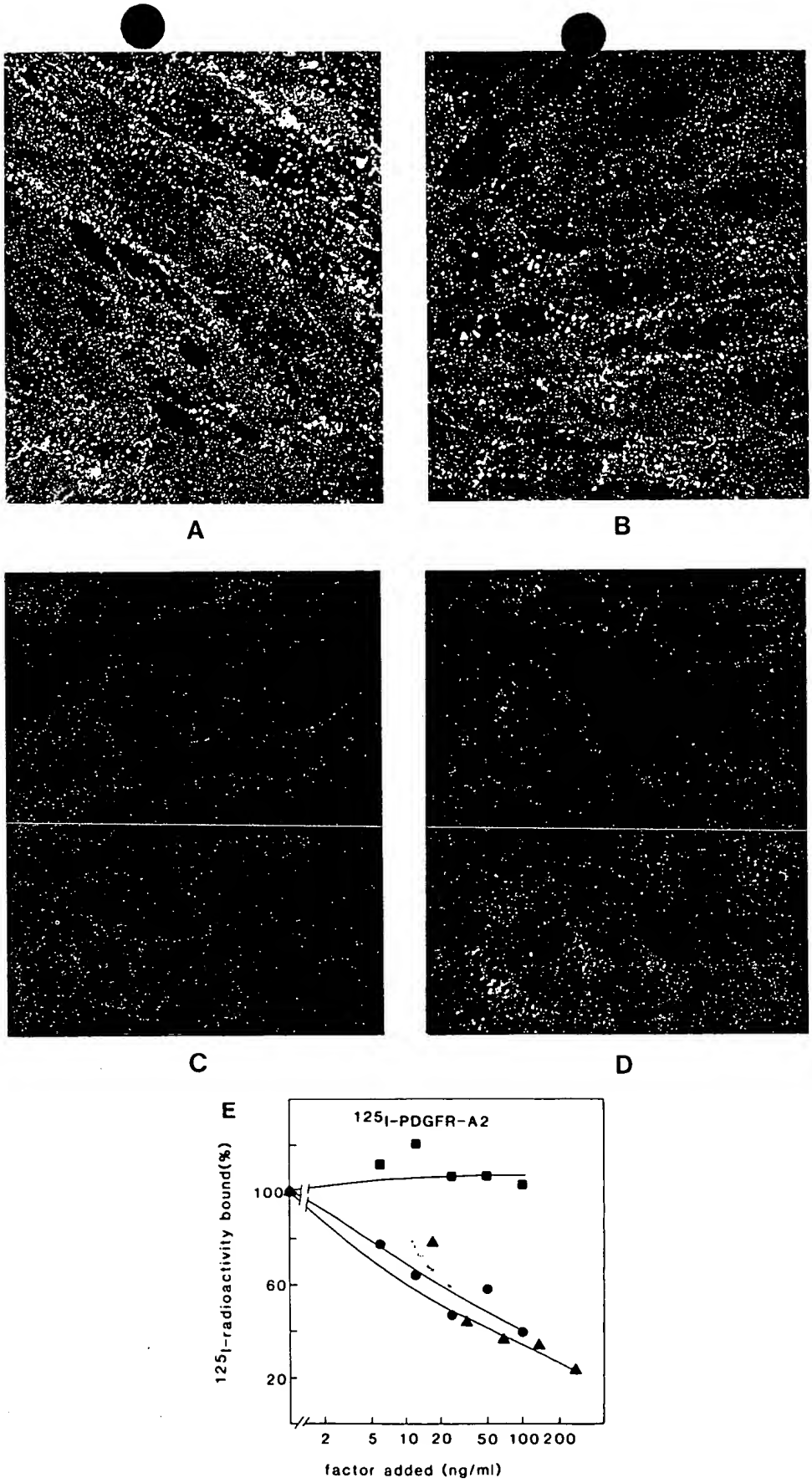

Fig. 5. Binding of monoclonal antibody PDGFR-B2 to human fibroblasts pretreated with various dimeric forms of PDGF. A PDGF receptor monoclonal antibody, PDGFR-B2, was used for immunofluorescence staining of human fibroblasts that had been preincubated at 37°C for 30 min with control buffer (A), or 100 ng/ml of PDGF-AA (B), PDGF-AB (C) or PDGF-BB (D). The binding of ^{125}I-labeled PDGFR-B2 at 0°C to cells that had been preincubated with increasing concentrations of PDGF-AB (●—●), PDGF-AA (■—■) or PDGF-BB (▲—▲) was also determined (E). 100% corresponds to 810 c.p.m. bound.

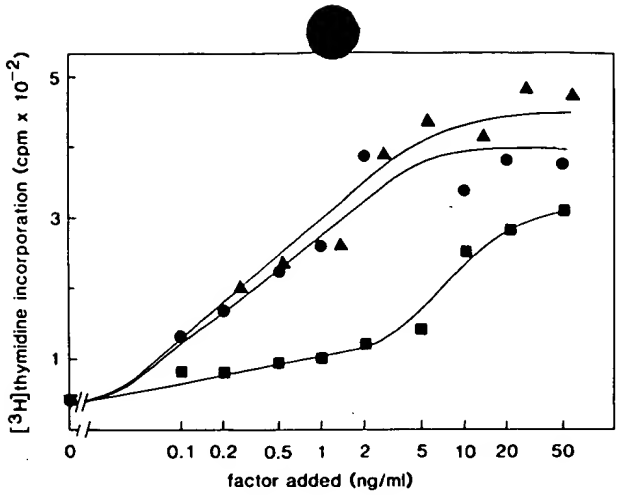

Fig. 6. Mitogenic activity of the different dimeric forms of PDGF. Increasing concentrations of PDGF-AB (●—●), PDGF-AA (■—■) or PDGF-BB (▲—▲) were assayed for the ability to stimulate incorporation of [^{3}H]TdR into human foreskin fibroblasts.

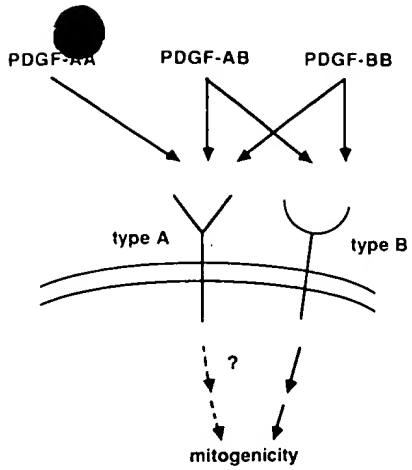

Fig. 7. Schematic illustration of the two different PDGF receptor types on human foreskin fibroblasts.

Binding of monoclonal antibody PDGFR-B2 to human fibroblasts pretreated with various dimeric forms of PDGF

Two monoclonal antibodies against the PDGF receptor purified from porcine uterus were recently established; both recognize the external domain of a human PDGF receptor (Rönnstrand *et al.*, 1988). One of these antibodies, PDGFR-B2, was investigated for its reactivity with A and B types of PDGF receptors. Human fibroblasts were preincubated for 60 min at 37°C with 100 ng/ml of PDGF-AA, PDGF-AB or PDGF-BB to down-regulate the corresponding receptors. As shown in Figure 5A, PDGFR-B2 gave a positive immunofluorescence staining on control cells. Preincubation with PDGF-AB (Figure 5C) or PDGF-BB (Figure 5D) quenched the immunofluorescence staining completely, but preincubation with PDGF-AA (Figure 5B) was essentially without effect. In order to quantify the binding of PDGFR-B2 antibody, the binding of ^{125}I-labeled PDGFR-B2 was determined on human fibroblasts preincubated at 37°C with different concentrations of the various dimeric forms of PDGF. PDGF-BB and PDGF-AB both caused half-maximal down-regulation at 30 ng/ml (Figure 5E). These results indicate that the PDGFR-B2 antibody recognizes the B type but not the A type of PDGF receptors, and that the previously purified PDGF receptor from porcine uterus (Rönnstrand *et al.*, 1987) is of B type.

Mitogenic activity of the different dimeric forms of PDGF

PDGF-AA, PDGF-AB and PDGF-BB were tested for their abilities to stimulate incorporation of [^{3}H]TdR into human fibroblasts. As shown in Figure 6, PDGF-AB and PDGF-BB were the most potent growth factors, half-maximal effects were achieved at about 1 ng/ml, whereas PDGF-AA was less potent, reaching the same degree of stimulation at 10 ng/ml. In addition, the maximal stimulation obtained with PDGF-AA was always lower than that of PDGF-BB or PDGF-AB.

Discussion

The present communication presents evidence that two distinct PDGF receptor classes exist that display differences in their binding of the three dimeric forms of PDGF. One receptor class, denoted type A, binds all dimeric forms of PDGF. The other receptor class, denoted type B, shows high affinity binding of PDGF-BB. It also binds PDGF-AB with lower affinity, but does not bind PDGF-AA. Figure 7 depicts schematically the two PDGF receptors and their ligand binding specificities.

A protein tyrosine kinase PDGF receptor (reviewed in Heldin and Rönnstrand, 1988) has been purified from 3T3 cells (Daniel *et al.*, 1985; Bishayee *et al.*, 1986) and porcine uterus (Rönnstrand *et al.*, 1987). cDNA for this receptor type (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1988) has been cloned, and expressed in CHO cells; the transfected cells bound [^{125}I]PDGF-BB with high affinity, [^{125}I]PDGF-AB with lower affinity, but not [^{125}I]PDGF-AA (Claesson-Welsh *et al.*, 1988), indicating that the cloned PDGF receptor is of the B type. The monoclonal antibodies that have been established against purified receptor preparations (Hart *et al.*, 1987; Rönnstrand *et al.*, 1988) are also directed against the B type receptor (Hart *et al.*, 1988; this work).

The size of the B type receptor is known from the predicted amino acid sequence of its cDNA clone (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1988), from the sizes in SDS/gel electrophoresis of components immunoprecipitated with specific antisera from metabolically labeled cells (Claesson-Welsh *et al.*, 1987; Hart *et al.*, 1987; Keatings and Williams, 1987), and of the purified receptor protein (Daniel *et al.*, 1985; Bishayee *et al.*, 1986; Rönnstrand *et al.*, 1987); the M_r of the polypeptide is 120 000 and the mature receptor, after post-translational modifications, has an apparent M_r in SDS/gel electrophoresis of 170 000–185 000. The determination of the size of the B type receptor by affinity labeling, 160–175 kd, after subtraction of the mol. wt of the ligand (Figure 4), conforms to these estimations. Previous estimates of the size of the PDGF receptor by affinity cross-linking using ^{125}I-labeled PDGF of unspecified dimeric forms yielded estimations of 163–185 kd (Glenn *et al.*, 1982; Heldin *et al.*, 1983; Williams *et al.*, 1984).

The major part of [^{125}I]PDGF-AA was cross-linked to form a complex of 140 kd, but a minor part also formed a complex of 175 kd (Figure 4). It is possible that the smaller molecule is formed from the larger by proteolysis; alternatively, the larger component reflects low affinity binding of [^{125}I]PDGF-AA to the B type receptor, or to another

component. Subtraction of the mol. wt of the ligand would yield a size for the type A receptor of 125 kd and of 160 kd for the larger molecule. Analysis by SDS–gel electrophoresis under non-reducing conditions revealed that the complex between [^{125}I]PDGF-AA and the receptor migrated slightly faster than under reducing conditions (125 versus 140 kd; not shown), indicating that the A type receptor, like the B type receptor, is a single chain protein which contains intra-chain disulphide bonds.

Certain growth factor receptor-like proto-oncogene products in this size range, with unknown ligands, have been described (reviewed in Westermark and Heldin, 1988). It remains to be seen whether any of them correspond to the A type PDGF receptor.

The affinity labeling experiment clearly indicated that the [^{125}I]PDGF-AB bound both to the A type (140 kd complex) and B type (175–190 kd complex) receptor. Preincubation of human fibroblasts with PDGF-AA, as expected, reduced the binding of the A type receptor. However, the binding to the B type receptor was also also reduced (Figure 4), suggesting that the presence of the A type receptor influenced the binding of [^{125}I]PDGF-AB to the B type receptor. The explanation for this finding is not known. PDGF induces dimerization of purified receptors after binding (unpublished observations). It is a possibility that the PDGF molecule might bind with higher affinity to one A and one B type receptor than to two receptors of the same type; removal of the A type receptor would then also decrease the binding of PDGF-AB to the B type receptor. Such a possibility is also supported by the fact that PDGF-AB was almost as potent as PDGF-BB in down-regulating the B type receptor on fibroblasts in spite of its lower affinity (Figure 2), as determined by the binding of the B type receptor antibody PDGFR-B2 (Figure 5).

In view of the fact that PDGF-AA, which seems to bind solely to the type A receptor, only has a limited mitogenic effect, it is likely that the B type receptor has a major role in the mediation of the mitogenic effect of PDGF. The low mitogenic effect of PDGF-AA might indicate that the A type receptor also mediates a mitogenic signal, albeit less efficiently. This possibility is also supported by the finding that PDGF-AB is as good a mitogen as PDGF-BB, in spite of the fact that PDGF-BB binds with a higher affinity to the B type receptor. It has not been excluded, however, that the mitogenic activity of PDGF-AA is mediated, directly or indirectly, via the B type receptor.

PDGF-AB and PDGF-BB, but not PDGF-AA, stimulate chemotaxis and actin reorganization leading to membrane ruffling (Nistér *et al.*, 1988; A.Siegbahn *et al.*, unpublished; K.Mellström *et al.*, unpublished). In fact, PDGF-AA inhibits PDGF-AB induced chemotaxis and, under certain conditions, membrane ruffling (Nistér *et al.*, 1988). This indicates that stimulation of chemotaxis and membrane ruffling are mediated by the B type receptor and that signals mediated by the interaction with the A type receptor, under certain conditions, may be inhibitory. The observation that PDGF-AA can transmodulate the EGF receptor (Nistér *et al.*, 1988), an effect which is mediated by activation of protein kinase C and phosphorylation of Thr-654 of the EGF receptor (Davis and Czech, 1985), suggests that the A receptor may be linked to the phosphatidylinositol cycle.

Expression of PDGF A chain mRNA and secretion of PDGF-AA have been found in many normal and transformed cell types. As PDGF-AA seems to mediate its effects via the A type receptor only, an important goal for future studies will be to characterize molecularly this receptor and identify the signals it transduces.

Materials and methods

PDGF

PDGF-AB was purified from human platelets as described (Heldin *et al.*, 1987). PDGF-AA and PDGF-BB were purified to apparent homogeneity from supernatants of yeast cells transfected with PDGF A chain and PDGF B chain constructs respectively (to be published). The concentrations of factors were determined by amino acid composition analysis. Some experiments were done with a partially purified batch of PDGF-BB.

PDGF-AA and PDGF-AB were ^{125}I-labeled with the chloramine T method (Hunter and Greenwood, 1982) to specific activities of 50 000 and 40 000 c.p.m./ng respectively. [^{125}I]PDGF-BB, labeled by the Bolton and Hunter method (1973) to a specific activity of 70 000 c.p.m./ng, was a kind gift of Amersham International.

Binding experiments

Binding experiments were performed on human foreskin fibroblasts (AG 1523, purchased from the Human Mutant Cell Repository, Camden, NJ) as described (Nistér *et al.*, 1984). Cells were grown in Linbro 12-well plates to confluence. Before binding, the cell cultures were rinsed once with 1 ml of binding buffer (PBS containing 1 mg/ml of bovine serum albumin, 0.01 mg/ml $CaCl_2.2H_2O$ and 0.01 mg/ml $MgSO_4.7H_2O$). Cell cultures were then incubated for 2 h at 0°C in 0.5 ml binding buffer containing 1–2 ng/ml of radiolabeled ligands and various concentrations of unlabeled ligands. Cultures were washed five times in ice-cold binding buffer; cells with associated ^{125}I-radioactivity were thereafter extracted by incubation for 30 min at room temperature in 0.5 ml Triton X-100, 20 mM Hepes, pH 7.4, 10% (v/v) glycerol, and determined in a gamma counter.

For down-regulation of the A type receptor, the fibroblast cultures were preincubated for 1 h at 37°C with 50 ng/ml of PDGF-AA. In some experiments, the cells were exposed for 1 min to binding buffer, supplemented with 20 mM acetic acid, pH 3.75, to dissociated receptor bound PDGF-AA at the cell surface. Cells were then washed in binding buffer at neutral pH.

Affinity cross-linking experiments

Human foreskin fibroblasts were grown to confluence on 6 cm Falcon dishes and incubated in the absence or presence of 50 ng/ml of PDGF-AA for 1 h at 37°C. After washing once in binding buffer, the cell cultures were incubated for 2 h at 0°C in 2 ml binding buffer containing 500 000 c.p.m. of ^{125}I-labeled PDGF-AA, PDGF-AB or PDGF-BB in the absence or presence of 50 ng/ml of the corresponding unlabeled ligand. After washing three times in ice-cold binding buffer and twice in PBS, cells were incubated for 20 min at 0°C in PBS containing 0.05 mM DSS. The cross-linking reaction was quenched by addition of 10 mM Tris base. After 10 min of incubation at 0°C, the cells were washed once in PBS containing 1 mM EDTA; the cells were then scraped off the plate with a rubber policeman in PBS containing 1 mM EDTA. Cells were pelleted by centrifugation for 5 min at 10 000 *g* and then solubilized for 30 min at 0°C in 1% Triton X-100, 20 mM Hepes, pH 7.4, 10% glycerol. After centrifugation for 5 min at 10 000 *g*, the supernatant was mixed with reducing SDS-sample buffer (Blobel and Dobberstein, 1975), heated at 95°C for 3 min, alkylated for 15 min at room temperature in 25 mM iodoacetamide, and analysed by SDS–gel electrophoresis.

SDS–gel electrophoresis

SDS–gel electrophoresis was performed as described (Blobel and Dobberstein, 1975) using gradient gels of 4–12% polyacrylamide.

Binding of monoclonal antibody

Human fibroblasts were grown to confluence on glass coverslips. After incubation for 1 h at 37°C in the absence or presence of 100 ng/ml of PDGF-AA, PDGF-AB or PDGF-BB, cells were washed once in serum-free Eagle's medium and incubated for 30 min at room temperature in serum-free Eagle's medium supplemented with 50 μg/ml of monoclonal antibody PDGFR-B2. Cells were then washed three times in serum-free Eagle's medium and incubated in a 1/40 dilution of FITC-labeled goat anti-mouse Ig (Beckton Dickinson) in serum-free Eagle's medium for 1 h at room temperature. After washing three times in PBS, cells were fixed in 2% *p*-formaldehyde in PBS. Cell cultures were inspected in a Nikon microscope equipped for epifluor-

escence and photographed using Kodak T-max film. Identical exposures were used to facilitate comparisons.

The binding of PDGFR-B2 to human fibroblasts was also determined by experiments utilizing ^{125}I-labeled PDGFR-B2 (labeled to a specific activity of 2200 c.p.m./ng by use of Iodobeads (Markwell, 1982). Confluent cell cultures in 12-well Linbro plates were preincubated in different concentrations of PDGF-AA, PDGF-AB or PDGF-BB for 1 h at 37°C, then washed once in 20 mM acetic acid, 0.15 M NaCl, 2.5 mg/ml bovine serum albumin, pH 3.75, to remove surface bound ligands, followed by one wash in binding buffer and then incubated for 2 h at 0°C in 0.5 ml binding buffer containing 1.1×10^6 c.p.m. [^{125}I]PDGFR-B2. Cells were then washed and cell associated radioactivity determined as described above. Non-specific binding was determined by inclusion of a 100-fold excess of unlabeled PDGFR-B2, and subtracted from the values of total binding.

Mitogenic assay

The mitogenic activity of PDGF-AB, PDGF-AA and PDGF-BB was determined as the ability to stimulate incorporation of [^{3}H]TdR into human fibroblasts (Betsholtz and Westermark, 1984).

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